StarGate®

The new dimension of combinatorial cloning

Instruction Manual

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Content

1 Introduction

Efficient procedures for functional expression, purification, detection, and immobilisation or separation of recombinant proteins – possibly in complex with cognate macromolecules – are of key importance in modern protein science. Many tools like various expression hosts (bacteria, yeast, insect and mammalian cells), promoters, affinity or fluorescent tags are currently available to fulfil these tasks. Due to the heterogenic nature of proteins, however, it is impossible to predict which combination of these tools will perform best in a certain situation. Therefore many have to be tried in order to identify an optimal solution.

To systemize and accelerate this initial search which is crucial for successful subsequent proteomic research, we have developed the StarGate system. StarGate offers rapid and highly efficient subcloning of an arbitrary gene – initially cloned in a Donor Vector - to fuse it in parallel with several different genetic surroundings via transfer into Acceptor Vectors to generate Destination Vectors (cf. section 5.1).

Key advantages of StarGate are

- minimal extra modification of the gene of interest due to short combinatorial sites
- inherent high level cloning efficiency due to a directed reaction (no equilibrium)
- availability of a multitude of combinatorial sites for combinatorial cloning

2 List of components

2.1 Donor Vector generation

2.1.1 StarGate Standard Entry Cloning Set

2.1.1.1 Supplied materials, long term storage conditions and recommendations

- Entry vector pENTRY-IBA10 (solution; 10 µl/reaction): -20°C. Spin briefly prior to use to recover contents. Store on ice during use.
- StarSolution E (solution, 20 reactions): -18°C to -20°C. Spin briefly prior to use to recover contents. Store on ice during use.
- Sequencing primer solution (10 µM; 45 µl for each forward (ENTRY-Primer-for) or reverse (ENTRY-Primer-rev) primer): -20°C. Spin briefly prior to use to recover contents.
- DNA Ruler (100 µl; manufactured by Fermentas): -20°C. Spin briefly prior to use to recover contents.
- Competent *E. coli* Top10: -80°C. Avoid any unnecessary warming of the cells by repacking or during storage until transformation. Even warming without thawing may already decrease ability of the cells for plasmid DNA uptake. Do not spin or vortex prior to use.

2.1.1.2 Additional materials required

- Primer set for amplification of the gene of interest and attachment of combinatorial sites
- *Pfu* DNA polymerase and corresponding PCR reagents
- Thermocycler
- Incubator
- Agarose gel electrophoresis equipment
- LB agar plates with 50 mg/L kanamycin and 50 mg/L X-gal
- *Xba*I/*Hin*dIII restriction endonucleases

2.1.2 StarGate Mutagenesis Entry Cloning Set

2.1.2.1 Supplied materials, long term storage conditions and recommendations

- Entry vector pENTRY-IBA20 (solution; 10 µl/reaction): -20°C. Spin briefly prior to use to recover contents. Store on ice during use.
- StarSolution M1 (solution, 5 reactions): -18°C to -20°C. Spin briefly prior to use to recover contents. Store on ice during use.
- StarSolution M2 (solution, 5 reactions): -18°C to -20°C. Spin briefly prior to use to recover contents. Store on ice during use.
- StarSolution M3 (solution, 5 reactions; has to be diluted with 4 µl M3-Diluent prior to use): -18°C to -20°C (prior and after dilution with M3-Diluent). Add 4 µl M3-Diluent, mix gently but thoroughly and spin briefly prior to use to recover contents. Store StarSolution M3 on ice during use. Mark vessel with a "+" after addition of M3-Diluent.
- M3-Diluent (solution): -18°C to -20°C. Spin briefly prior to use to recover contents.

- Sequencing primer solution (10 µM; 20 µl for each forward (ENTRY-Primer-for) or reverse (ENTRY-Primer-rev) primer): -20°C. Spin briefly prior to use to recover contents.
- DNA Ruler (25 µl; manufactured by Fermentas): -20°C. Spin briefly prior to use to recover contents.
- Competent *E. coli* Top10: -80°C. Avoid any unnecessary warming of the cells by repacking or during storage until transformation. Even warming without thawing may already decrease ability of the cells for plasmid DNA uptake. Do not spin or vortex prior to use.

2.1.2.2 Additional materials required

- Primer set for amplification of the gene of interest (attachment of combinatorial sites and introduction of site directed mutations)
- *Pfu* DNA polymerase and corresponding PCR reagents
- Thermocycler
- Incubator
- Agarose gel electrophoresis equipment
- LB agar plates with 50 mg/L kanamycin and 50 mg/L X-gal
- *Xba*I/*Hin*dIII restriction endonucleases

2.2 Destination Vector generation

2.2.1 StarGate Transfer Cloning Set

2.2.1.1 Supplied materials, long term storage conditions and recommendations

- Acceptor Vector (solution; 10 µl/reaction):): -18°C to -20°C. Spin briefly prior to use to recover contents. Store on ice during use.
- StarSolution A1 (solution, 20 reactions): -18°C to -20°C. Spin briefly prior to use to recover contents. Store on ice during use.
- StarSolution A2 (solution, 20 reactions): -18°C to -20°C. Spin briefly prior to use to recover contents. Store on ice during use.
- StarSolution A3 (solution, 20 or 30 reactions, has to be diluted with 13 µl or 20 µl, respectively, A3-Diluent prior to use): -18°C to -20°C (prior and after dilution with A3-Diluent). Add 13 µl or 20 µl A3-Diluent (the size of the content (20 or 30 reactions) is indicated on the label), mix gently but thoroughly and spin briefly prior to use to recover contents. Store StarSolution A3 on ice during use. Mark vessel with a "+" after addition of A3-Diluent.
- A3-Diluent (solution): -18°C to -20°C. Spin briefly prior to use to recover contents.
- Competent *E. coli* Top10: -80°C. Avoid any unnecessary warming of the cells by repacking or during storage until transformation. Even warming without thawing may already decrease ability of the cells for plasmid DNA uptake. Do not spin or vortex prior to use.

2.2.1.2 Additional materials required

- Incubator
- LB agar plates with 100 mg/L ampicillin and 50 mg/L X-gal

2.3 Control reactions

2.3.1 StarGate Control Set (Cat. no. 5-1604-000)

2.3.1.1 Supplied materials, long term storage conditions and recommendations

- Control PCR-fragment (solution; phosphorylated blunt end PCR-fragment, 720 bp, 10 nM, 30 µl): -18°C to -20°C. Spin briefly prior to use to recover contents.
- Control Donor vector (solution; Donor vector with 720 bp DNA insert, 2 ng/µl, 25 µl): -18°C to -20°C. Spin briefly prior to use to recover contents.

3 StarGate procedure

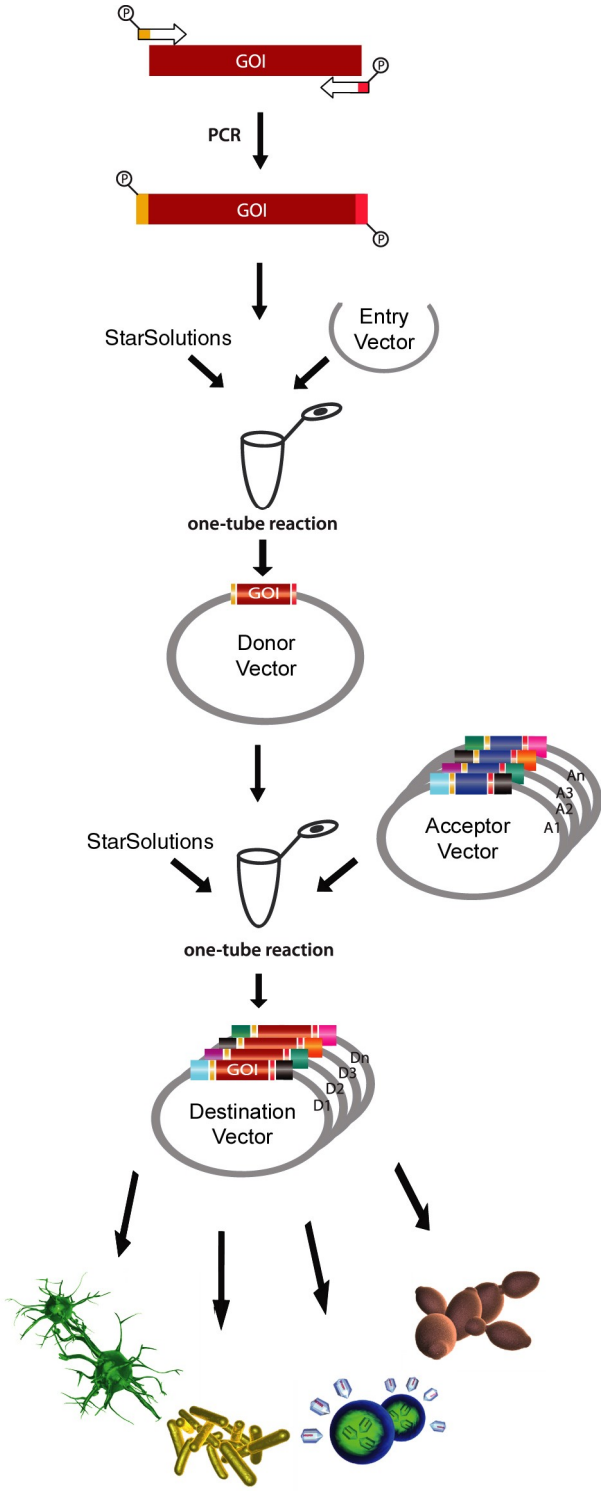
3.1 Workflow

Step 1: Donor Vector generation

In a first step, the gene of interest (GOI) is equipped at both ends with combinatorial sites (4 bases) by PCR and is inserted into an Entry Vector by a simple one-tube reaction. The opened Entry Vector contributes the recognition sites and brings them into operative linkage with the combinatorial sites for the highly specific StarGate gene transfer process from Step 2.

Step 2: Destination Vector generation

After sequence confirmation, the resulting Donor Vector is the origin for the highly parallel subcloning of GOI into a multitude of Acceptor Vectors, each providing a different genetic surrounding like host specific promoters and different purification tags by a second simple one-tube reaction. The resulting Destination Vectors are then transformed into the corresponding host cells for further experiments.



3.2 Step 1: Donor Vector generation

There are two possibilities to generate a Donor vector.
1) Wild type GOI's are inserted into the Entry Vector by using the Standard Entry Cloning Set after simple PCR amplification of GOI (see 3.2.1).
2) If, however, mutated derivatives of the GOI are to be expressed, then a modified PCR process together with the Mutagenesis Entry Cloning Set is used to generate the Donor Vector (see 3.2.2)

3.2.1 Wild type GOI with Standard Entry Cloning Set

The gene of interest (GOI) has to be equipped in a first step at both termini with combinatorial sites, which will serve as recombination sites for the transfer of the GOI into the Acceptor Vectors. This is conveniently performed by PCR (Figure 1).

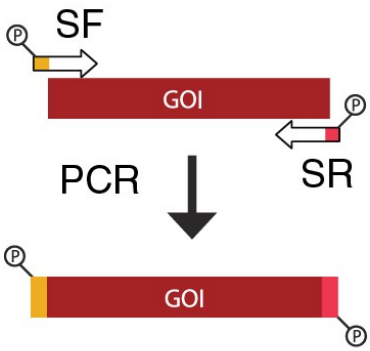


Figure 1
The standard forward primer (SF) starts with the upstream combinatorial site 5'-AATG followed by nucleotides complementary to the antisense strand of GOI determining the amino acid sequence following the Met start codon. The Met start codon is provided by the combinatorial site. The standard reverse primer (SR) starts with the reverse complement 5'-TCCC of the downstream combinatorial site (5'-GGGA) directly followed by nucleotides coding for the last amino acids of the GOI and complementary to the reverse of the sense strand of GOI.

Example:
The GOI has the following sequence (leave out the Met start codon and the stop codon):

```
5'-TTGACCTGCAACAGCCTGTCCATCGGAACATTGCGATGCATAGCC-3'
3'-AACTGGACGTTGTCGGACAGGTAGCCTTGTAACGCTACGTATCGG-5'
   LeuThrCysAsnSerLeuSerIleGlyThrLeuArgCysIleAla
```

Appropriate primers have to be designed (use StarPrimer D'Signer 2.0 or refer to 4.1.1.1) so that the resulting PCR product will additionally include the combinatorial sites. In this example, the PCR product would then have the following sequence:

```
5'P-AATGTTGACCTGCAACAGCCTGTCCATCGGAACATTGCGATGCATAGCCGGGA-3'
 3'-TTACAACTGGACGTTGTCGGACAGGTAGCCTTGTAACGCTACGTATCGGCCCT-P5'
    MetLeuThrCysAsnSerLeuSerIleGlyThrLeuArgCysIleAlaGly
```

The Met start codon is reconstituted by the upstream combinatorial site (italics and bold) while the stop codon is replaced by a glycin "GGG" codon via the downstream combinatorial site (italics and bold).

Important:

- Use a proof reading DNA polymerase like *Pfu* (Fermentas) that minimizes the risk of mutations and generates a PCR product with blunt ends.

- 5' phosphorylated primers have to be used for PCR of GOI or, alternatively, the PCR product may be phosphorylated by T4 polynucleotide kinase prior to purification which is, however, less efficient.

In a second step, the phosphorylated PCR product is inserted into an Entry Vector which thereby results in a Donor Vector (Figure 2).

This is achieved by mixing the Entry Vector with the PCR product, adding a StarSolution and incubating the resulting mixture for one hour.

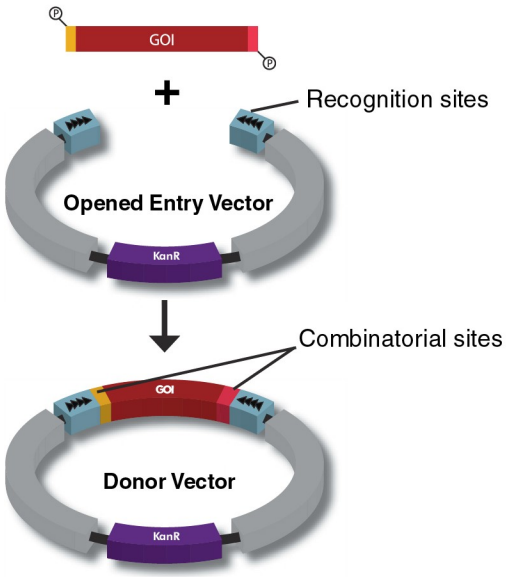


Figure 2
Insertion of GOI into an Entry Vector to create a Donor Vector. The PCR amplified GOI with the combinatorial sites (4 bases) at both ends is inserted into an Entry Vector. The opened Entry Vector contributes the recognition sites and brings them into operative linkage with the combinatorial sites for the highly specific StarGate gene transfer process.

Insertion of PCR product/GOI in the Donor Vector can be checked by restriction analysis. The orientation of Donor Vector inserted GOI is not relevant for subsequent oriented transfer of the gene into an Acceptor vector. As PCR, however, may lead to mutations and to improper product ends, it is recommended to confirm GOI and flanking sequences by sequencing using ENTRY-Primer-for and/or ENTRY-Primer-rev included in the kit.

3.2.2 Mutated GOI with Mutagenesis Entry Cloning Set

To generate a StarGate compatible Donor vector with a modified sequence of the GOI, a special PCR-based procedure can be used. The design of appropriate PCR primers is simplified by the free software StarPrimer D'Signer 2.0. For this purpose, the sequences of the initial, non-mutated gene of interest (as it is present in the template DNA for PCR) as well as of the mutated gene have both to be copied, without start and stop codon, and pasted into the corresponding menu field of the program. 2 to 4 primers are designed by the program with which 1 to 2 PCR's have to be performed using the initial non-mutated gene of interest as template. As a proof reading polymerase (*Pfu*) has to be used for PCR it is essential to use 3' phosphorothioate (PTO) protected primers.

In a second step, the 2 PCR products are inserted in a directed manner into an Entry Vector which thereby results in a Donor Vector (Figure 3).

This is achieved by mixing the 2 PCR products with the Entry Vector, adding StarSolutions M1, M2 and M3 and incubating the resulting mixture for 1 hour prior to transforming *E. coli*.

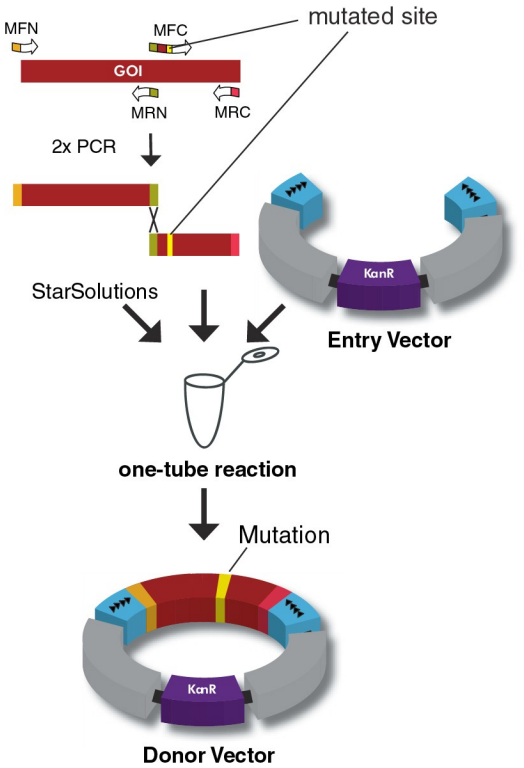


Figure 3
Directed assembly of 2 PCR products with an Entry Vector to create a Donor Vector with a mutated GOI.

Insertion of PCR products/mutated GOI in the Donor Vector can be checked by restriction analysis. As PCR may lead to non-desired mutations, it is recommended to confirm mutated GOI sequence by Donor Vector sequencing using ENTRY-Primers included in the kit.

3.3 Step 2: Destination Vector generation

Acceptor vectors provide the desired genetic surrounding (i.e. tag, promoter, signal sequence and the like, cf. 5.1). The appropriate Acceptor Vector is mixed with the Donor Vector and with StarSolutions and the desired Destination vector will form after a short incubation. *E. coli* is transformed with the mixture and plated on LB agar plates containing ampicillin and X-gal. Desired Destination Vectors including GOI will generate white colonies while non-desired Acceptor Vectors will generate blue colonies (Figure 4).

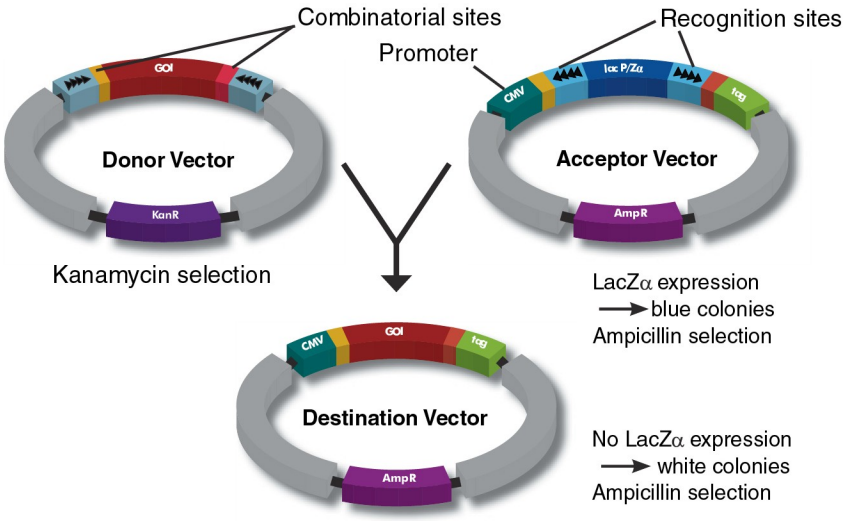


Figure 4

E. coli is transformed with the mixture potentially including all 4 possible vectors (Donor Vector, Acceptor Vector, Destination Vector and By product [not shown]). Due to selection on ampicillin plates, Donor Vector and By product – which provide a kanamycin resistance gene only – will not enable growth of *E. coli*. Acceptor Vector and Destination Vectors, however, enable growth due to the encoded ampicillin resistance genes. Colonies carrying a Destination Vector can nevertheless be differentiated from colonies carrying an Acceptor Vector. The Acceptor Vector carries the LacZα gene and, therefore, produces blue colonies on X-gal containing plates while LacZα has been replaced by GOI in the Destination Vector which, therefore, generates white colonies. The Destination Vector of this example puts GOI under control of the CMV promoter enabling GOI expression in mammalian cells and fuses a tag to the C-terminal end of GOI expression product. The recognition sites providing specific gene transfer at the combinatorial sites are eliminated in the Destination Vector recombination product. This drives the reaction until almost each Acceptor Vector has been equipped with a GOI.

4 StarGate protocols/recommendations

4.1 Step 1: Donor vector generation with Standard Entry Cloning Set

4.1.1 PCR to amplify and equip GOI with combinatorial sites

4.1.1.1 Primer design

If, e.g., subsequent sequence would represent a GOI (start and stop codon have to be left out)

```
5′-TTGACCTGCAACAGCCTGTCCATCGGAACATTGCGATGCATAGCC-3′
3′-AACTGGACGTTGTCGGACAGGTAGCCTTGTAACGCTACGTATCGG-5′
   LeuThrCysAsnSerLeuSerIleGlyThrLeuArgCysIleAla
```

then the following **5′ phosphorylated primers** have to be designed for PCR to equip GOI with combinatorial sites:

```
5′P-AATGTTGACCTGCAACAGCCTGTC-3′ (SF: standard forward primer)
       ||||||||||||||||||
     5′-TTGACCTGCAACAGCCTGTCCATCGGAACATTGCGATGCATAGCC-3′
     3′-AACTGGACGTTGTCGGACAGGTAGCCTTGTAACGCTACGTATCGG-5′
                               ||||||||||||||||||
(SR: standard reverse primer) 3′-CTTGTAACGCTACGTATCGGCCCT-P5′
```

Initial hybridizing region (marked with ||) should have a melting temperature between 60 and 63 °C when calculated following the rule of thumb that each A:T-pair contributes 2 °C and each G:C-pair 4 °C.

Important:

- We recommend using primers that are phosphorylated synthetically which is more efficient and will give better blunt end cloning results than post synthetic phosphorylation via T4 polynucleotide kinase and ATP.

- Since a proof reading polymerase like *Pfu* has to be used for PCR, **3′ phosphorothioate protected primers** have to be used. Otherwise, proof reading activity may degrade the primers from the 3′ end during PCR thereby impairing annealing and consequently yield of PCR product.

5′ end of the SF primer (standard forward primer) is elongated by an additional AATG quadruplet to generate the upstream combinatorial site and
5′ end of the SR primer (standard reverse primer) is elongated by an additional TCCC quadruplet to generate the downstream combinatorial site.

The desired PCR product then has the following structure and will be phosphorylated:

```
5′P-AATGTTGACCTGCAACAGCCTGTCCATCGGAACATTGCGATGCATAGCCGGGA-3′
 3′-TTACAACTGGACGTTGTCGGACAGGTAGCCTTGTAACGCTACGTATCGGCCCT-P5′
```

4.1.1.2 PCR amplification of the GOI

Perform PCR with *Pfu* DNA polymerase. Use of a proof reading polymerase is essential. Mix the following reagents in a 500 µl reaction tube:

		final concentration:	
dNTP (10 mM each)	1 µl	200 µM	
Phos. forward primer (10 µM)	2,5 µl	500 nM	
Phos. reverse primer (10 µM)	2,5 µl	500 nM	
10x buffer (supplier)	5 µl		
Template DNA	X µl	20 to 200 pg/µl	(plasmid DNA)
		0,1 to 1 ng/µl	(cDNA library)
H_2O	ad 50 µl		

Overlay the sample with 50 µl mineral oil (alternatively, use a heated lid when available) and heat the sample at 94 °C for 3 min. Add 1 µl *Pfu* DNA polymerase (2,5 U/µl) and start temperature cycling.

Anneal and denature for 30 sec or 1 min. Since the rate of synthesis of *Pfu* is significantly slower than that of *Taq*, the duration of the DNA synthesis step should be doubled when using *Pfu* in comparison to protocols referring to the use of *Taq* polymerase (further information can be obtained from the respective manufacturer of *Pfu* DNA polymerase). PCR annealing should be performed at 55 °C as a starting point when primer design had occurred following paragraph 4.1.1.1 or by StarPrimer D'Signer 2.0.

If plasmid DNA with an already cloned gene is used as a template, 15 to 20 cycles are usually sufficient, while 30 to 40 cycles are recommended for cDNA libraries as template. Generally, the number of cycles should be kept as low as possible in order to minimize the possibility of incorporation of base substitutions. A final 60°C incubation of 5 min should be performed in order to obtain full length products. Samples are stored at 4°C until further purification.

Essential parameters for optimization are the annealing temperature, the duration of synthesis and the template concentration.

When PCR reaction produced a single product of the expected size, purification using a clean up kit to remove *Pfu* polymerase and primers may be sufficient to proceed to Donor Vector generation reaction. It is, however, recommended to isolate the PCR product by preparative gel electrophoresis which may reduce efforts for identifying a correct clone after Donor Vector generation reaction. Elute PCR product from purification column with water.

Quantify PCR product by gel electrophoresis through band intensity comparison with the DNA Ruler (manufactured by Fermentas) included in the kit (Figure 5). Applying 2 different amounts of PCR product in separate lanes is recommended to find a band of equal intensity with a band of DNA Ruler which has to be applied on the same gel as internal standard for exact quantification.

Apply 5 - 10 µl DNA Ruler per lane.

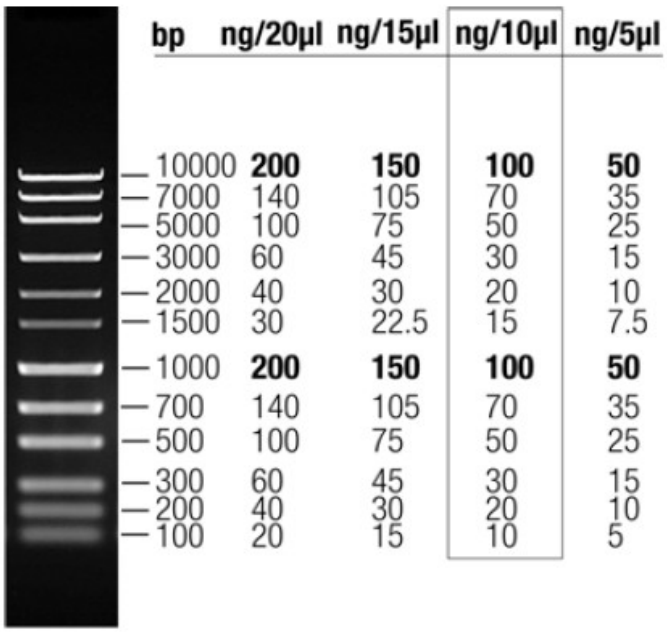


Figure 5
DNA Ruler is a molecular size standard where each band represents a defined amount of linear DNA.

Determine PCR product concentration and dilute the PCR product to between 4 and 16 nM with water (corresponds to 1.4-5.6 ng/µl for a 0.5 kb fragment, 2.8-11.2 ng/µl for a 1 kb fragment, 4.2-16.8 ng/µl for a 1.5 kb fragment, 5.6-22.4 ng/µl for a 2 kb fragment, 7-28 ng/µl for a 2.5 kb fragment, etc.).

4.1.2 Reaction for Donor Vector generation (wild type GOI)

1. Add the following reagents to the supplied reaction tube containing the Entry Vector pENTRY-IBA10 (10 µl)
   Add:

PCR product (between 4 and 16 nM) from 4.1.1.2	14 µl
StarSolution E	1 µl

2. Close the reaction vessel thoroughly, mix gently and incubate for 1 h at 22 °C.

3. Thaw a vial of supplied competent *E. coli* cells on ice.

4. After incubation, pipet off an aliquot of 10 µl from the reaction mixture (25 µl) from step 2 and add it to the thawed competent *E. coli* cells. Continue incubation of the residual reaction mixture (15 µl) over night at 16 °C as backup solution if no colonies could be generated by the primary reaction.

5. Mix gently (do not vortex) and incubate subsequently for 30 min on ice.

6. Mix gently (do not vortex) and incubate subsequently for 5 min at 37 °C.

7. Mix gently (do not vortex) and incubate subsequently 2-5 min on ice.

8. Add 900 µl LB medium and shake for 45 min at 37 °C.
   Caution: This incubation step is necessary to express kanamycin resistance prior to plating on kanamycin plates for selection.

9. Plate 100 µl on LB agar containing 50 mg/L kanamycin and 50 mg/L X-gal.

10. Centrifuge the residual 900 µl cell mixture for 30 sec in a microfuge, resuspend the cell sediment with 100 µl LB medium and plate the whole amount on plates as above.

11. Incubate plates over night at 37 °C.

4.1.3 Donor Vector identification (wild type GOI)

Pick 10 white colonies and perform DNA mini preparation.

Perform analytical *Xba*I/*Hin*dIII restriction. A fragment having the length of the PCR product from 4.1.1.1 plus 40 bases is expected as long as no *Xba*I and/or *Hin*dIII restriction sites are present in GOI. Select a putatively correct clone and confirm sequence via Donor Vector forward and reverse sequencing. Appropriate primers are included in the kit (ENTRY-Primer-for and ENTRY-Primer-rev). The region flanking the combinatorial sites should have the sequence:

```
TCTAGAAAGGAACGTCTCCAATG-GOI-GGGAGGAGACGAAGGAAAAGCTT
 XbaI                                          HindIII

or, in case of inverted insertion of GOI,

AAGCTTTTCCTTCGTCTCCAATG-GOI-GGGAGGAGACGTTCCTTTCTAGA
HindIII                                        XbaI
```

Sequencing is recommended as PCR may lead to mutations and to improper product ends.

Dilute 1 µg of the verified Donor Vector plasmid DNA with water to 500 µl (final concentration is 2 ng/µl) and store at – 20 °C.

4.2 Step 1: Donor vector generation with Mutagenesis Entry Cloning Set

4.2.1 PCR's to create the mutated GOI sequence

Primer design is achieved by the StarPrimer D'Signer software 2.0. Typically, the software will determine 4 primers, i.e. MFN (mutagenesis forward primer N), MRN (mutagenesis reverse primer N), MFC (mutagenesis forward primer C) and MRC (mutagenesis reverse primer C). In exceptional cases – if mutations to be introduced are close to one end of GOI – 2 primers will be determined only, i.e. MF (mutagenesis forward primer) and MR (mutagenesis reverse primer).

Amplify the GOI by 2 separate PCR's using the primer pairs MFN/MRN and MFC/MRC (or alternatively by one PCR with MF/MR). Perform PCR and determine PCR product concentration as described in paragraph 4.1.1.2.

In case of 2 PCR products (MFN/MRN and MFC/MRC), dilute each PCR product to 4 nM with water (corresponds to 1.4 ng/µl for a 0.5 kb fragment, 2.8 ng/µl for a 1 kb fragment, 4.2 ng/µl for a 1.5 kb fragment, 5.6 ng/µl for a 2 kb fragment, 7 ng/µl for a 2.5 kb fragment, etc.).

In case of 1 PCR product (MF/MR), dilute the PCR product to 2 nM with water (corresponds to 0.7 ng/µl for a 0.5 kb fragment, 1.4 ng/µl for a 1 kb fragment, 2.1 ng/µl for a 1.5 kb fragment, 2.8 ng/µl for a 2 kb fragment, 3.5 ng/µl for a 2.5 kb fragment, etc.).

4.2.2 Reaction for Donor Vector generation (mutated GOI)

1. Add the following reagents to the supplied reaction tube containing the Entry Vector pENTRY-IBA20 (10 µl)
   Add:

Water diluted PCR product (MFN/MRN, 4 nM) from 4.2.1	6 µl
Water diluted PCR product (MFC/MRC, 4 nM) from 4.2.1	6 µl
StarSolution M1	1 µl
StarSolution M2	1 µl
StarSolution M3 (**supplemented with M3-Diluent, see 2.1.2.1**)	1 µl

   When only 1 PCR product has to be prepared (cf. 4.2.1), use 12 µl of the 2 nM solution of this PCR product.

2. Close the reaction vessel thoroughly, mix gently and incubate for 1 h at 30 °C.

3. Thaw a vial of supplied competent *E. coli* cells on ice.

4. After incubation, pipet off an aliquot of 10 µl from the reaction mixture (25 µl) from step 2 and add it to the thawed competent *E. coli* cells. Continue incubation of the residual reaction mixture (15 µl) in the refrigerator at 2-8°C for backup purposes.

5. Mix gently (do not vortex) and incubate subsequently for 30 min on ice.

6. Mix gently (do not vortex) and incubate subsequently for 5 min at 37 °C.

7. Mix gently (do not vortex) and incubate subsequently 2-5 min on ice.

8. Add 900 µl LB medium and shake for 45 min at 37 °C.
   Caution: This incubation step is necessary to express kanamycin resistance prior to plating on kanamycin plates for selection.

9. Plate 100 µl on LB agar containing 50 mg/L kanamycin and 50 mg/L X-gal.

10. Centrifuge the residual 900 µl cell mixture for 30 sec in a microfuge, resuspend the cell sediment with 100 µl LB medium and plate the whole amount on plates as above.

11. Incubate plates over night at 37 °C.

4.2.3 Donor Vector identification (mutated GOI)

Pick 5 white colonies and perform DNA mini preparation.

Perform *Xba*I/*Hin*dIII restriction. A fragment having the length of mutated GOI plus 40 bases is expected as long as no *Xba*I and/or *Hin*dIII restriction sites are present in GOI. Select a putatively correct clone and confirm sequence via Donor Vector forward and reverse sequencing primers included in the kit (ENTRY-Primer-for and ENTRY-Primer-rev). The combinatorial site flanking region should have the sequence:

```
TCTAGAAAGGAACGTCTCCAATG-mutGOI-GGGAGGAGACGAAGGAAAAGCTT
 XbaI                                                HindIII
```

Sequencing is recommended as PCR may lead to mutations and to improper product ends.

Dilute 1 µg of the verified Donor Vector plasmid DNA with water to 500 µl (final concentration is 2 ng/µl) and store at – 20 °C.

4.3 Step 2: Destination Vector generation

4.3.1 GOI transfer reaction

1. Add the reagents below to the supplied reaction tube containing the appropriate Acceptor Vector (10 µl)
   Add:

Diluted Donor Vector solution (2 ng/µl; see 4.1.3 or 4.2.3)	12 µl
StarSolution A1	1 µl
StarSolution A2	1 µl
StarSolution A3 (**supplemented with A3-Diluent, see 2.2.1.1**)	1 µl

2. Close the reaction vessel thoroughly, mix gently and incubate at 30 °C for 1 h.

3. Thaw a vial of supplied competent *E. coli* cells on ice.

4. After incubation, pipet off an aliquot of 10 µl from the reaction mixture (25 µl) from step 2 and add it to the thawed competent *E. coli* cells. Continue incubation of the residual reaction mixture (15 µl) in the refrigerator at 2-8°C for backup purposes.

5. Mix gently (do not vortex) and incubate subsequently for 30 min on ice.

6. Mix gently (do not vortex) and incubate subsequently for 5 min at 37 °C.

7. Mix gently (do not vortex) and incubate subsequently 2-5 min on ice.

8. Plate 10 µl (mixed with 90 µl LB medium) and 100 µl on LB agar containing 100 mg/L ampicillin and 50 mg/L X-gal.

9. Incubate plates over night at 37 °C.

4.3.2 Destination Vector identification

Pick 3 white colonies and perform DNA mini preparation.

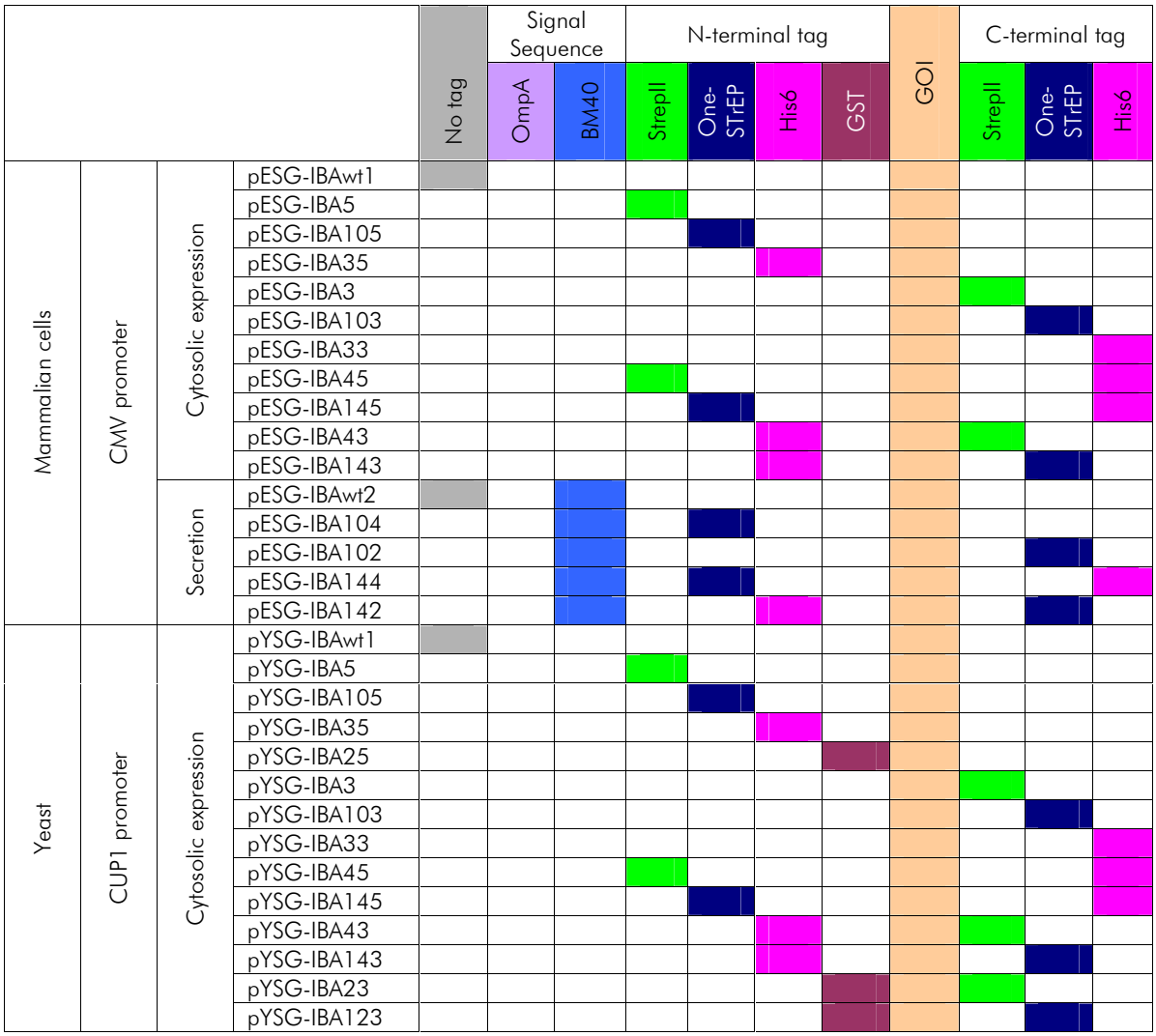
pASG-IBA, pPSG-IBA, pESG-IBA and pYSG-IBA have *Xba*I/*Hin*dIII restriction sites that flank the expression cassette and, therefore, may be used for confirmation of GOI integration. For exact calculation of expected restriction fragment length please refer to the appropriate Acceptor Vector data sheet.

5 StarGate Acceptor Vector collection

5.1 Overview

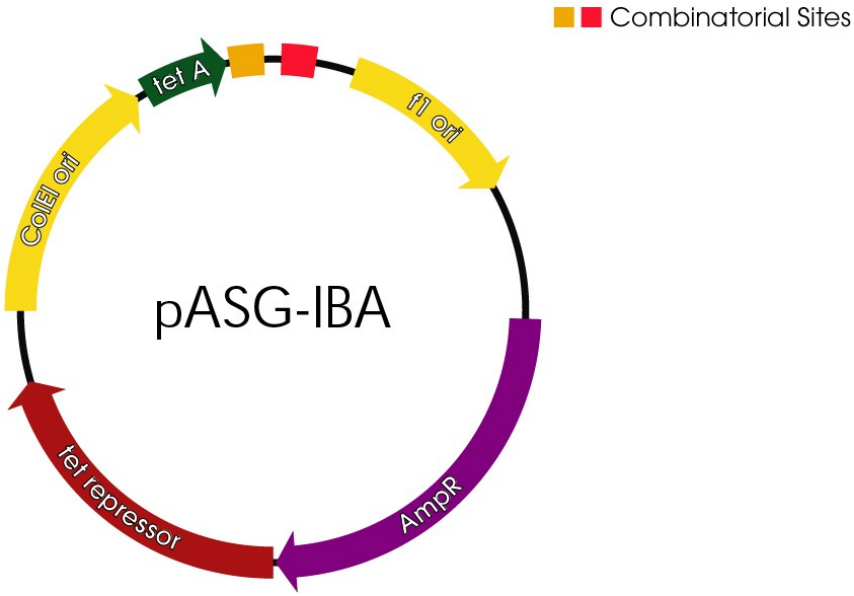
					Signal Sequence		N-terminal tag					C-terminal tag		
				No tag	OmpA	BM40	StrepII	One-STrEP	His6	GST	GOI	StrepII	One-STrEP	His6
E. coli	Tet promoter	Cytosolic expression	pASG-IBAwt1											
			pASG-IBA5											
			pASG-IBA105											
			pASG-IBA35											
			pASG-IBA25											
			pASG-IBA3											
			pASG-IBA103											
			pASG-IBA33											
			pASG-IBA45											
			pASG-IBA145											
			pASG-IBA43											
			pASG-IBA143											
			pASG-IBA23											
			pASG-IBA123											
		Periplasmic expression	pASG-IBAwt2											
			pASG-IBA4											
			pASG-IBA104											
			pASG-IBA44											
			pASG-IBA144											
			pASG-IBA2											
			pASG-IBA102											
	T7 promoter	Cytosolic expression	pPSG-IBAwt1											
			pPSG-IBA5											
			pPSG-IBA105											
			pPSG-IBA35											
			pPSG-IBA25											
			pPSG-IBA3											
			pPSG-IBA103											
			pPSG-IBA33											
			pPSG-IBA45											
			pPSG-IBA145											
			pPSG-IBA43											
			pPSG-IBA143											
			pPSG-IBA23											
			pPSG-IBA123											

StarGate Acceptor Vector collection, overview, continued



StarGate Acceptor Vector collection will be constantly expanded. Please inquire at info@iba-go.com when a suitable Acceptor Vector for the desired application cannot be found as this Table may not represent the most recent state.

5.2 pASG-IBA vector description

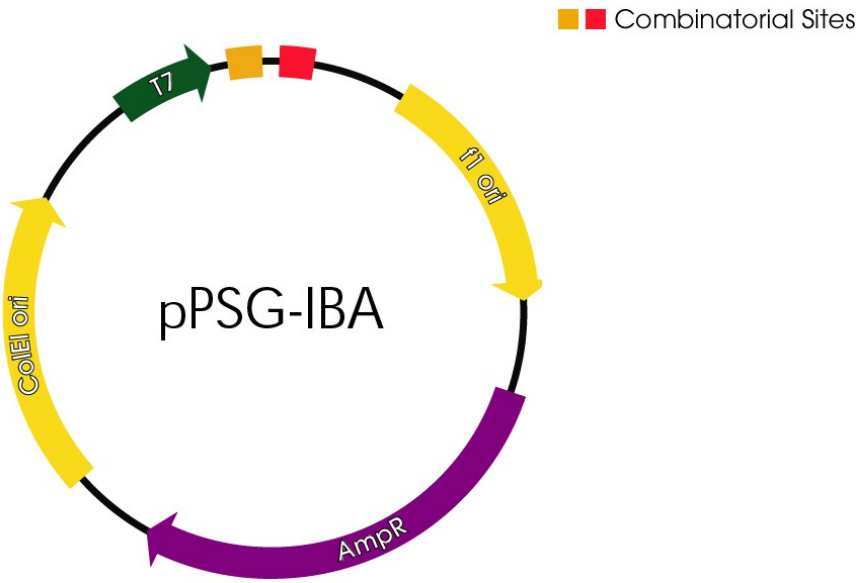


The production of a heterologous protein is often accompanied by an impaired growth of *E. coli* cells. Consequently, regulation of heterologous biosynthesis is generally recommended by the use of a promoter whose activity can be blocked by a repressor. If the foreign protein is cytotoxic, even the production of minute quantities can result in a dramatic selection against the *E. coli* cells which harbor the expression plasmid. In such cases, tight repression of the promoter is required. Synthesis of the gene product is then switched on in a controlled manner simply by adding a chemical inducer. pASG-IBA vectors which are similar to pASK-IBA vectors carry the promoter/operator region from the *tet*A resistance gene and are the optimal solution for such an inducible expression system (Skerra, 1994). The strength of the *tet*A promoter is comparable with that of the *lac*-UV5 promoter. Some vectors carry the ompA signal sequence for secretion of the recombinant protein into the periplasmic space which is crucial for functional expression of proteins with structural disulfide bonds.

The *tet* promoter can be fully induced by adding anhydrotetracycline at a concentration that is not antibiotically effective (200 ng/ml). The constitutive expression of the *tet* repressor gene, which is also encoded on the expression plasmids, guarantees the repression of the promoter in the absence of the inducer. In a Western blot, no expression is detectable under these conditions (Skerra, 1994). In contrast to the *lac* promoter, which is susceptible to catabolite repression (cAMP-level, metabolic state) and chromosomally encoded repressor molecules, the *tet*A promoter/operator is not coupled to any cellular regulation mechanisms. Therefore, when using the *tet* system, there are basically no restrictions in the choice of culture medium or *E. coli* expression strain. For example, glucose minimal media and even the bacterial strain XL1-Blue, which carries an episomal copy of the tetracycline resistance gene, can be used for expression.

Note: Please refer to the corresponding data sheet for detailed information on a given vector

5.3 pPSG-IBA vector description

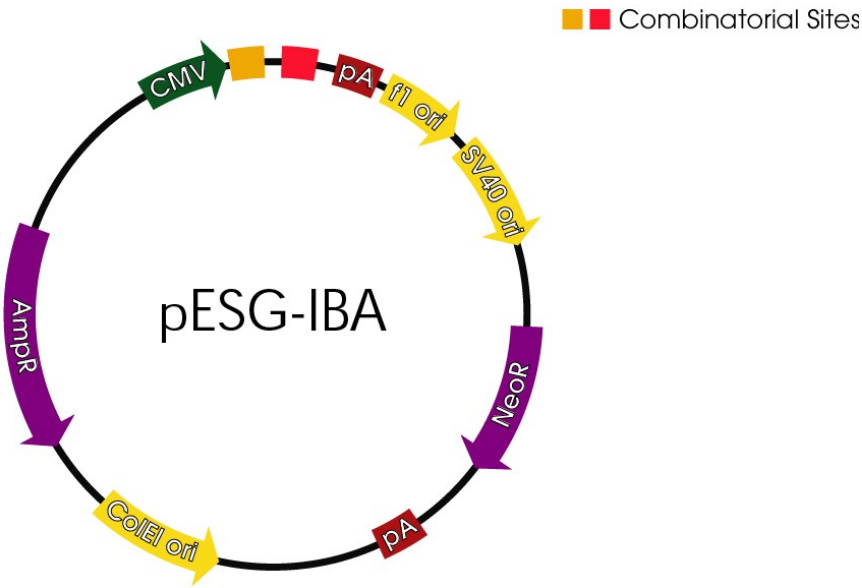


pPSG-IBA vectors which are similar to pPR-IBA vectors use the T7 promoter and T7 RNA polymerase for high-level transcription of the gene of interest (Studier et al., 1990). As the T7 promoter is stronger than the *tet* promoter, pPSG-IBA vectors are recommended in cases where expression with the *tet* promoter does not lead to significant yields of the recombinant protein. In other cases, strong T7 expression may cause insoluble inclusion bodies. In such cases the *tet* promoter might be a good alternative when expression of soluble protein is desired.

Expression of the target genes is induced by providing a source of T7 RNA polymerase in the *E. coli* host cell. This is accomplished by using, e.g., an *E. coli* host which contains a chromosomal copy of the T7 RNA polymerase gene (e.g. BL21(DE3) which has the advantage to be deficient of *lon* and *ompT* proteases). The T7 RNA polymerase gene is under control of the lacUV5 promoter which can be induced by addition of IPTG.

Note: Please refer to the corresponding data sheet for detailed information on a given vector

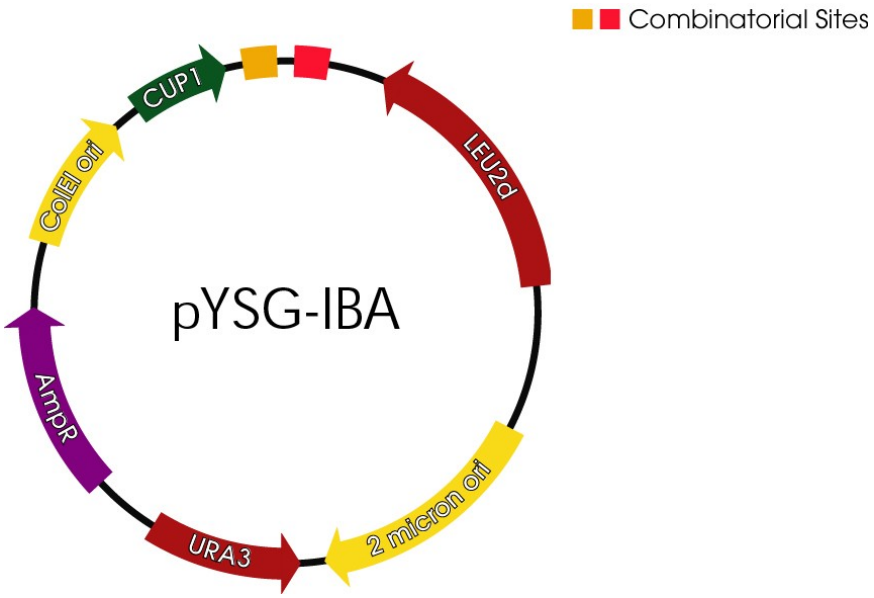
5.4 pESG-IBA vector description



pESG-IBA vectors are designed for high-level constitutive expression of recombinant proteins in a wide range of mammalian host cells through the human cytomegalovirus immediate-early (CMV) promoter (Boshart et al., 1985; Nelson et al., 1987). To prolong expression in transfected cells, the vector will replicate in cell lines that are latently infected with SV40 large T antigen (e.g. COS7). In addition, Neomycin resistance gene allows direct selection of stable cell lines. Propagation in *E. coli* is supported by a ColEI ori and the ampicillin resistance gene. Finally, some vectors carry the BM40 signal sequence for secretion of the recombinant protein into the medium.

Note: Please refer to the corresponding data sheet for detailed information on a given vector

5.5 pYSG-IBA vector description



pYSG-IBA expression vectors are designed for high-level expression of recombinant proteins in yeast. Cloned genes are under the control of the Cu^{++}-inducible CUP1 promoter which means that expression is induced upon addition of copper sulfate. pYSG-IBA vectors favour correct protein folding and the production of soluble proteins — inclusion bodies rarely form.

In addition, all vectors include the *E. coli* ampicillin resistance gene and the yeast selectable markers leu2-d (a LEU2 gene with a truncated, but functional promoter) and URA3. Vectors including the leu2-d marker are maintained at high copy number to provide enough gene products from the inefficient promoter for cell survival during growth selection in minimal medium lacking leucine (Macreadie et al., 1991; Gietz & Sugino, 1989).

Note: Please refer to the corresponding data sheet for detailed information on a given vector

6 StarGate Acceptor Vector expression cassettes

6.1 pASG-IBA expression cassettes

```
pASG-IBAwt1:  AATG-GOI-GGGAGCTAA
                M  -POI-G  S  *

pASG-IBAwt2:  ATGAAAAAGACA-OMPA-GCGCAGGCCGCAATG-GOI-GGGAGCTAA
              M  K  K  T  -OMPA-A  Q  A  A  M  -POI-G  S  *
                                    ↑

pASG-IBA2:    ATGAAAAAGACA-OMPA-GCGCAGGCCGCAATG-GOI-GGGAGCGCTTGGAGCCACCCGCAGTTCGAAAAATAA
              M  K  K  T  -OMPA-A  Q  A  A  M  -POI-G  S  A  W  S  H  P  Q  F  E  K  *
                                    ↑

pASG-IBA3:    AATG-GOI-GGGAGCGCTTGGAGCCACCCGCAGTTCGAAAAATAA
                M  -POI-G  S  A  W  S  H  P  Q  F  E  K  *

pASG-IBA4:    ATGAAAAAGACA-OMPA-GCGCAGGCCGCAATGGAGTCATCCTCAATTCGAAAAATCCGGAATG-GOI-GGGAGCTAA
              M  K  K  T  -OMPA-A  Q  A  A  M  A  S  A  W  S  H  P  Q  F  E  K  S  G  M  -POI-G  S  *
                                    ↑

pASG-IBA5:    ATGGCTAGCGCATGGAGTCATCCTCAATTCGAAAAATCCGGAATG-GOI-GGGAGCTAA
              M  A  S  A  W  S  H  P  Q  F  E  K  S  G  M  -POI-G  S  *

pASG-IBA23:   ATGTCCCCTATA-GST-CCTCCAAAAATGTCCGGAGGTGGCGGTGGGAGCCTGGAAGTTCTGTTCCAGGGGCCAATG-GOI-GGGAGCGCTTGGAGCCACCCG
              M  S  P  I  -GST-P  P  K  M  S  G  G  G  G  S  L  E  V  L  F  Q  G  P  M  -POI-G  S  A  W  S  H  P
                                                                                    ↑

              CAGTTCGAAAAATAA
              Q  F  E  K  *

pASG-IBA25:   ATGTCCCCTATA-GST-CCTCCAAAAATGTCCGGAGGTGGCGGTGGGAGCCTGGAAGTTCTGTTCCAGGGGCCAATG-GOI-GGGAGCTAA
              M  S  P  I  -GST-P  P  K  M  S  G  G  G  G  S  L  E  V  L  F  Q  G  P  M  -POI-G  S  *
                                                                                    ↑

pASG-IBA33:   AATG-GOI-GGGAGCGCTTCACCATCACCATCACCATTAA
                M  -POI-G  S  A  H  H  H  H  H  H  *

pASG-IBA35:   ATGGCTAGCCATCACCATCACCATCACTCCGGAATG-GOI-GGGAGCTAA
              M  A  S  H  H  H  H  H  H  S  G  M  -POI-G  S  *

pASG-IBA43:   ATGGCTAGCCATCACCATCACCATCACTCCGGAATG-GOI-GGGAGCGCTTGGAGCCACCCGCAGTTCGAAAAATAA
              M  A  S  H  H  H  H  H  H  S  G  M  -POI-G  S  A  W  S  H  P  Q  F  E  K  *
```

pASG-IBA expression cassettes, continued

pASG-IBA expression cassettes, continued

6.2 pPSG-IBA expression cassettes

```
pPSG-IBAwt1: AATG-GOI-GGGAGCTAA
             M  -POI-G  S  *

pPSG-IBA3:   AATG-GOI-GGGAGCGCTTGGAGCCACCCGCAGTTCGAAAAATAA
             M  -POI-G  S  A  W  S  H  P  Q  F  E  K  *

pPSG-IBA5:   ATGGCTAGCGCATGGAGTCATCCTCAATTCGAAAAATCCGGAATG-GOI-GGGAGCTAA
             M  A  S  A  W  S  H  P  Q  F  E  K  S  G  M  -POI-G  S  *

pPSG-IBA23:  ATGTCCCCTATA-GST-CCTCCAAAAATGTCCGGAGGTGGCGGTGGGAGCCTGGAAGTTCTGTTCCAGGGGCCAATG-GOI-GGGAGCGCTTGGAGCCACCCGCAG
             M  S  P  I  -GST-P  P  K  M  S  G  G  G  G  S  L  E  V  L  F  Q  G  P  M  -POI-G  S  A  W  S  H  P  Q
                                                                  ↑
             TTCGAAAAATAA
             F  E  K  *

pPSG-IBA25:  ATGTCCCCTATA-GST-CCTCCAAAAATGTCCGGAGGTGGCGGTGGGAGCCTGGAAGTTCTGTTCCAGGGGCCAATG-GOI-GGGAGCTAA
             M  S  P  I  -GST-P  P  K  M  S  G  G  G  G  S  L  E  V  L  F  Q  G  P  M  -POI-G  S  *
                                                                  ↑

pPSG-IBA33:  AATG-GOI-GGGAGCGCTCACCATCACCATCACCATTAA
             M  -POI-G  S  A  H  H  H  H  H  H  *

pPSG-IBA35:  ATGGCTAGCCATCACCATCACCATCACTCCGGAATG-GOI-GGGAGCTAA
             M  A  S  H  H  H  H  H  H  S  G  M  -POI-G  S  *

pPSG-IBA43:  ATGGCTAGCCATCACCATCACCATCACTCCGGAATG-GOI-GGGAGCGCTTGGAGCCACCCGCAGTTCGAAAAATAA
             M  A  S  H  H  H  H  H  H  S  G  M  -POI-G  S  A  W  S  H  P  Q  F  E  K  *

pPSG-IBA45:  ATGGCTAGCGCATGGAGTCATCCTCAATTCGAAAAATCCGGAATG-GOI-GGGAGCGCTCACCATCACCATCACCATTAA
             M  A  S  A  W  S  H  P  Q  F  E  K  S  G  M  -POI-G  S  A  H  H  H  H  H  H  *

pPSG-IBA103: AATG-GOI-GGGAGCGCTTGGAGCCACCCGCAGTTCGAAAAAGGTGGAGGTTCTGGCGGTGGATCGGGAGGTTCAGCGTGGAGCCACCCGCAGTTCGAGAAATAA
             M  -POI-G  S  A  W  S  H  P  Q  F  E  K  G  G  G  S  G  G  G  S  G  G  S  A  W  S  H  P  Q  F  E  K  *
```

pPSG-IBA expression cassettes, continued

6.3 pESG-IBA expression cassettes

pESG-IBA expression cassettes, continued

```
pESG-IBA105:  ATGGCTAGCGCATGGAGTCATCCTCAATTCGAGAAAGGTGGAGGTTCTGGCGGTGGATCGGGAGGTTCAGCGTGGAGCCACCCGCAGTTCGAA
              M  A  S  A  W  S  H  P  Q  F  E  K  G  G  G  S  G  G  G  S  G  G  S  A  W  S  H  P  Q  F  E
              AAATCCGGAATG-GOI-GGGAGCTAA
              K  S  G  M  -POI-G  S  *

pESG-IBA142:  ATGAGGGGCCTGG-BM40-GCTCTGGCAGCAATGGCTAGCCATCACCATCACCATCACTCCGGAATG-GOI-GGGAGCGCTTGGAGCCACCCGCAGTTCGAAAAA
              M  R  A  W  -BM40-A  L  A  A  M  A  S  H  H  H  H  H  H  S  G  M  -POI-G  S  A  W  S  H  P  Q  F  E  K
                                         ↑
              GGTGGAGGTTCTGGCGGTGGATCGGGAGGTTCAGCGTGGAGCCACCCGCAGTTCGAGAAATAA
              G  G  G  S  G  G  G  G  S  G  G  S  A  W  S  H  P  Q  F  E  K  *

pESG-IBA143:  ATGGCTAGCCATCACCATCACCATCACTCCGGAATG-GOI-GGGAGCGCTTGGAGCCACCCGCAGTTCGAAAAAGGTGGAGGTTCTGGCGGTGGATCGGGAGGT
              M  A  S  H  H  H  H  H  H  S  G  M  -POI-G  S  A  W  S  H  P  Q  F  E  K  G  G  G  S  G  G  G  S  G  G
              TCAGCGTGGAGCCACCCGCAGTTCGAGAAATAA
              S  A  W  S  H  P  Q  F  E  K  *

pESG-IBA144:  ATGAGGGGCCTGG-BM40-GCTCTGGCAGCAATGGAGTCATCCTCAATTCGAGAAAGGTGGAGGTTCTGGCGGTGGATCGGGAGGTTCAGCG
              M  R  A  W  -BM40-A  L  A  A  M  A  S  A  W  S  H  P  Q  F  E  K  G  G  G  S  G  G  G  S  G  S  A
                                         ↑
              TGGAGCCACCCGCAGTTCGAAAAATCCGGAATG-GOI-GGGAGCGCTCACCATCACCATCACCATTAA
              W  S  H  P  Q  F  E  K  S  G  M  -POI-G  S  A  H  H  H  H  H  H  *

pESG-IBA145:  ATGGCTAGCGCATGGAGTCATCCTCAATTCGAGAAAGGTGGAGGTTCTGGCGGTGGATCGGGAGGTTCAGCGTGGAGCCACCCGCAGTTCGAA
              M  A  S  A  W  S  H  P  Q  F  E  K  G  G  G  S  G  G  G  S  A  W  S  H  P  Q  F  E
              AAATCCGGAATG-GOI-GGGAGCGCTCACCATCACCATCACCATTAA
              K  S  G  M  -POI-G  S  A  H  H  H  H  H  H  *
```

6.4 pYSG-IBA expression cassettes

pYSG-IBA expression cassettes, continued

```
pYSG-IBA123:  ATGTCCCCTATA-GST-CCTCCAAAAATGTCCGGAGGTGGCGGTGGGAGCCTGGAAGTTCTGTTCCAGGGGCCAATG-GOI-GGGAGCGCTTGGAGCCACCCGCAG
              M  S  P  I  -GST-P  P  K  M  S  G  G  G  G  S  L  E  V  L  F  Q  G  P  M  -POI-G  S  A  W  S  H  P  Q
                                                                                        ↑
                    TTCGAAAAAGGTGGAGGTTCTGGCGGTGGATCGGGAGGTTCAGCGTGGAGCCACCCGCAGTTCGAGAAATAA
                    F  E  K  G  G  G  S  G  G  G  S  G  G  S  A  W  S  H  P  Q  F  E  K  *

pYSG-IBA143:  ATGGCTAGCCATCACCATCACCATCACTCCGGAATG-GOI-GGGAGCGCTTGGAGCCACCCGCAGTTCGAAAAAGGTGGAGGTTCTGGCGGTGGATCGGGAGGT
              M  A  S  H  H  H  H  H  H  S  G  M  -POI-G  S  A  W  S  H  P  Q  F  E  K  G  G  G  S  G  G  G  S  G  G
                    TCAGCGTGGAGCCACCCGCAGTTCGAGAAATAA
                    S  A  W  S  H  P  Q  F  E  K  *

pYSG-IBA145:  ATGGCTAGCGCATGGAGTCATCCTCAATTCGAGAAAGGTGGAGGTTCTGGCGGTGGATCGGGAGGTTCAGCGTGGAGCCACCCGCAGTTCGAA
              M  A  S  A  W  S  H  P  Q  F  E  K  G  G  G  S  G  G  G  S  G  G  S  A  W  S  H  P  Q  F  E
                    AAATCCGGAATG-GOI-GGGAGCGCTCACCATCACCATCACCATTAA
                    K  S  G  M  -POI-G  S  A  H  H  H  H  H  H  *
```

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